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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

1300-1-009

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

09/856864

INTERNATIONAL APPLICATION NO.  
PCT/GB99/03979

INTERNATIONAL FILING DATE  
29 November 1999

PRIORITY DATE CLAIMED  
27 November 1998

**TITLE OF INVENTION**

**DNA ENCODING FUSION PROTEINS WHICH ARE SPECIFICALLY CLEAVABLE BY COPPER (II) IONS**

**APPLICANT(S) FOR DO/EO/US**

David Paul HUMPHREYS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☒ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Six (6) Sheets of Formal Drawings (Figs. 1-9); International Search Report; Written Opinion; International Preliminary Examination Report

**EXPRESS MAIL "MAILING CERTIFICATE NO.": EL758522818 US DATE OF DEPOSIT: MAY 25, 2001**

U.S. APPLICATION NO. (If known, see 37 CFR 1.507) <b>097/853864</b>	INTERNATIONAL APPLICATION NO. PCT/GB99/03979	ATTORNEY'S DOCKET NUMBER 1300-1-009
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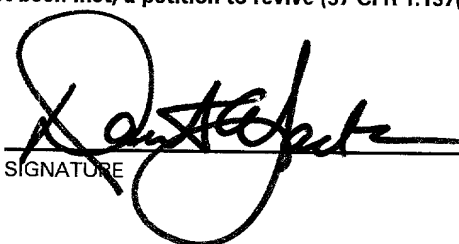
17. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... \$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$710.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$1,000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$ 100.00 <div style="text-align: right; margin-top: 10px;"> <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b> </div>				<div style="text-align: right; font-size: 1.2em;">\$ 860.00</div>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
Claims	Number Filed	Number Extra	Rate		
Total Claims	11 -20 =	0	X \$18.00		
Independent Claims	2 -3 =	0	X \$80.00	<div style="text-align: right; font-size: 1.2em;">\$ 270.00</div>	
Multiple dependent claim(s) (if applicable)			+ \$270.00		
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<div style="text-align: right; font-size: 1.2em;">\$ 1,130.00</div>	
Reduction for 1/2 for filing by small entity, if applicable.				<div style="text-align: right; font-size: 1.2em;">\$</div>	
<b>SUBTOTAL =</b>				<div style="text-align: right; font-size: 1.2em;">\$ 1,130.00</div>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				<div style="text-align: right; font-size: 1.2em;">\$ .00</div>	
<b>TOTAL NATIONAL FEE =</b>				<div style="text-align: right; font-size: 1.2em;">\$ 1,130.00</div>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				<div style="text-align: right; font-size: 1.2em;">\$</div>	
<b>TOTAL FEES ENCLOSED =</b>				<div style="text-align: right; font-size: 1.2em;">\$ 1,130.00</div>	
				Amount to be:	\$
				refunded	\$
				charged	\$

- a. ☒ A check in the amount of \$ 1,130.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 11-1153 in the amount of \$          to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 11-1153. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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 SIGNATURE

NAME  
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 REGISTRATION NUMBER

**EXPRESS MAIL "MAILING CERTIFICATE NO.": EL758522818 US DATE OF DEPOSIT: MAY 25, 2001**

09/856864

25 MAY 2001

PATENT  
1300-1-009

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS : David Paul Humphreys  
APPLICATION NO. : PCT/GB99/03979  
FILED : 29 November 1999  
FOR : DNA ENCODING FUSION PROTEINS WHICH ARE  
SPECIFICALLY CLEAVABLE BY COPPER (II) IONS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
BOX PCT  
WASHINGTON, D.C. 20231

Sir:

Prior to calculating the fees pursuant to the entry into the National Phase of the above-identified Application, please amend the claims as follows:

IN THE CLAIMS:

Please amend Claims 4 and 6 as follows:


4. (Amended) A vector comprising DNA according to any of Claims 1 to 3.
6. (Amended) A process for the production of a desired protein in native form comprising 1) expressing the desired protein as a fusion protein in a host cell according to Claim 5 in which the desired protein is expressed as one of two polypeptides joined by a site specifically cleavable by copper (II) ions, 2) cleaving the fusion protein with copper (II) ions and 3) recovering the desired cleaved protein in native form.

REMARKS

The above amendments are submitted herewith to conform the claims more closely to U.S. practice.

Entry of the foregoing amendments and early and favorable processing in the National Phase before the United States Patent and Trademark Office is courteously solicited.

Respectfully submitted,



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Enclosure: Marked-Up Version of Claims

**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In The Claims:**

Claims 4 and 6 have been amended as follows:

4. (Amended) A vector comprising DNA according to any of Claims 1 to [Claim] 3.

6. (Amended) A process for the production of a desired protein in native form

comprising 1) expressing the desired protein as a fusion protein in a host cell according to Claim 5 in which the desired protein is expressed as one of two polypeptides joined by a site specifically cleavable by copper (II) ions, 2) cleaving the fusion protein with copper (II) ions and 3) recovering the desired cleaved protein in native form.

## DNA ENCODING FUSION PROTEINS WHICH ARE SPECIFICALLY CLEAVABLE BY COPPER (II) IONS

5 This invention relates to recombinant fusion proteins and to methods of cleaving such fusion proteins to yield a desired protein in native form.

10 In order to aid intra-cellular solubility, purification and detection, many proteins of biotechnological interest are expressed as fusion proteins including: galactosidase, IgG binding peptide, glutathione S-transferase, maltose binding protein, His-tag, myc-tag, cellulose binding domain, calmodulin binding peptide, FLAG-tag, and Strep-tag [Casadaban MJ *et al*, (1983); Nilsson B *et al*, (1987); Smith DB and Johnson KS, (1988); di Guan C *et al*, (1988); Hochuli E *et al*, (1988); Dreher ML *et al*, (1991); Ong E *et al*, 1991; Stofko-Hahn RE *et al*, (1992); Brizzard BL *et al*, (1994); and 15 Schmidt TGM and Skerra A, (1994). For full details of the publications referred to herein, see the section of the specification headed "REFERENCES"].

20 It is often undesirable for the fusion partner to remain associated with the protein of interest after purification since it may interfere with protein function, structural analysis, or be immunogenic if the protein is administered *in vivo*. Peptide sites cleaved by specific proteases such as Factor Xa, TEV, Genenase I, thrombin, enterokinase and 3C can be introduced between the two protein domains to enable cleavage of the two 25 domains [Nagai K and Thøgersen HC, (1984); Dougherty WG *et al*, (1988); Carter P and Wells JA, (1987); McKenzie KR *et al*, (1991); LaVallie ER *et al*, (1993); Walker PA *et al*, (1994)]. However, these enzymes can be expensive, have variable effectiveness due to differences in the sequences surrounding the target site and accessibility of the site to a 30 large enzyme, and require a further purification step to remove the proteolytic enzyme from the sample.

35 The upper hinge tetrapeptide sequence <sup>N</sup>DKTH<sup>C</sup> was identified as the site of cleavage of a highly purified recombinant humanised  $\gamma$ 1 antibody by traces of  $\text{Cu}^{2+}$  [Smith M A *et al*, (1996)]. The sequence was cleaved between the Lys and Thr residues. The degree of cleavage increased with

increasing temperature, length of time, and pH of incubation, and also with increasing amounts of available  $\text{Cu}^{2+}$  ions. The  $\text{NDKTHC}$  sequence was also cleaved by  $\text{Cu}^{2+}$  when present in a small peptide [Smith MA *et al*, (1996)].

5

We have now devised an improved cleavage linker for use in the construction of recombinant DNA sequences which code for fusion proteins. The linker codes for a copper(II) site comprising a sequence of four different amino acids.

10

Thus according to one aspect of the invention we provide DNA coding for a fusion protein comprising two polypeptides joined by a cleavage site which does not naturally join the polypeptides and which is specifically cleavable by copper(II) ions.

15

For the avoidance of doubt, the term "which does not naturally join" is used herein to exclude proteins known in nature to include an amino acid sequence which is cleavable by copper(II) ions, for example as described hereinafter.

20

The invention also includes a vector comprising DNA coding for a fusion protein comprising two polypeptides joined by a cleavage site which does not naturally join the polypeptides and which is specifically cleavable by copper(II) ions, and host cells transformed with such a vector. The vector may be in particular an expression vector as more particularly described hereinafter. Host cells include any procaryotic or eucaryotic cells, especially bacterial, yeast, animal, e.g. mammalian, insect and plant cells.

25

Furthermore the invention includes a process for the production of a desired protein in native form, comprising 1) expressing the desired protein as a fusion protein in a host cell transformed with a vector according to the invention in which the desired protein is expressed as one of two polypeptides joined by a site specifically cleavable by copper(II) ions, 2) cleaving the fusion protein with copper(II) ions and 3) recovering the desired cleaved protein in native form. The fusion protein produced in this process is novel and forms a further aspect of the invention.

30

35

In all the aspects of the invention described herein the cleavage site may be any tetrapeptide which may be cleaved by copper(II) ions and includes in particular the tetrapeptide <sup>N</sup>DKTH<sup>C</sup> as described herein and functional equivalents thereof, for example where one or more functionally equivalent amino acids is included as alternatives to those shown. Suitable substitutions include those amino acids which are generally recognised as conservative changes for D, K, T and H, for example E for D, R for K, S for T and Y for H. Single, double or other multiple substitutions may be made.

5      Examples of particularly useful alternatives to the <sup>N</sup>DKTH<sup>C</sup> site include <sup>N</sup>DRSH<sup>C</sup>, <sup>N</sup>EKSH<sup>C</sup> and especially <sup>N</sup>DKSH<sup>C</sup>. DNA coding for the peptide <sup>N</sup>DKTH<sup>C</sup> includes that described in the experimental section hereinafter and functional equivalents thereof, for example due to the degeneracy of the genetic code. DNA coding for any of the above functional equivalents

10      of <sup>N</sup>DKTH<sup>C</sup> is similarly readily available.

All aspects of the invention are widely applicable to the production of any desired protein, and the various terms "polypeptide", "fusion protein" and "protein" as used herein are to be understood to include any protein,

20      polypeptide, peptide and fragments thereof from any procaryotic, eucaryotic or synthetic source, except where indicated otherwise. Particular examples include mammalian polypeptides and proteins, including enzymes; serum proteins, especially antibodies; cytokines; hormones; growth factors; receptors; structural proteins; and precursors

25      and fragments thereof.

The fusion proteins according to the invention may be produced using standard recombinant DNA techniques involving the expression of the proteins by a host cell. Isolated DNA coding for each protein may be

30      introduced for example into any suitable expression vector by operatively linking the DNA to any necessary expression control elements therein and transforming any suitable procaryotic or eucaryotic host cell with the vector using well known procedures, for example as described hereinafter in the experimental section. For creation of the DNA coding for the fusion

35      protein, appropriate DNA coding for the protein of interest and its fusion



partner may be fused via an oligonucleotide sequence coding for the copper cleavage site using conventional annealing procedures to ensure that the correct reading frame is maintained in the vector. Where other potential copper cleavage sites are identified in the starting DNA, these  
 5 can be removed using standard mutagenesis techniques for example as described in the Examples hereinafter.

The expresssion of the fusion protein during the culture of the host cell may be monitored using standard assay procedures and at any  
 10 appropriate time during or after the culture the protein may be isolated from the host cells and/or culture medium using conventional protein purification techniques such as chromatography and filtration.

Once obtained, the fusion proteins according to the invention may be  
 15 cleaved using copper(II) ions to yield the desired recombinant protein. The cleavage reaction may be performed using any source of copper(II) ions, for example a copper(II) salt, e.g.  $\text{CuCl}_2$ , in a suitable solvent, for example an aqueous buffered solvent, at an appropriate pH and temperature. The precise reaction conditions, including the quantity of copper used, will  
 20 depend on the nature and quantity of the fusion protein and the cleavage products and can advantageously be determined empirically for each protein, for example as described in the experimental section hereinafter. When an aqueous buffer is used, the buffer may advantageously be a Tris buffer. The production of the desired protein may be monitored during the  
 25 cleavage reaction using any appropriate assay, based for example on the activity, immunoreactivity and/or size of the protein. The desired protein may be recovered after the cleavage reaction using standard protein isolation and purification techniques, for example as just described.

30 The following Figures are referred to in the Examples below. In the Figures:

**Figure 1** Effect of buffer type on efficiency of cleavage of 'Null 2 FLAG'  $\text{F(ab')}_2$  by  $\text{Cu}^{2+}$

35 Coomassie stained non-reducing SDS-PAGE using 4-20% Tris-glycine gels. All lanes contain  $1\mu\text{g}$  protein equivalents from cleavage reactions

using 50mM of buffer at pH8.5 (except MES which was pH5.5) for 16h at 62°C. The size and migration of molecular weight markers are shown on the left in kDa. The cleavage reactions in lanes 1 and 2 were Tris buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively;

5 Lanes 3 and 4 were Bicine buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively;

Lanes 5 and 6 were Tricine buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively;

10 Lanes 7 and 8 were CHES buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively;

Lanes 9 and 10 were HEPES buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively;

Lanes 11 and 12 were MES buffered with 1mM and 0.1mM  $\text{Cu}^{2+}$  respectively.

15

**Figure 2** Effect of pH on efficiency of cleavage by Cu(II)

**Figure 3** Demonstration of cleavage of FLAG tail and effect of  $[\text{Cu}^{2+}]$

20 Non-reducing SDS-PAGE using 4-20% Tris-glycine gels. All lanes contain 1µg protein equivalents (unless indicated) from cleavage reactions using 50mM of buffer at pH9.0 (except MES which was pH5.5) for 24h at 62°C. Lanes 1 to 8 are Coomassie stained. The size and migration of molecular weight markers are shown on the left in kDa. The cleavage reactions in lanes 1 to 5 were Tris buffered with 5mM, 2.5mM, 1mM, 0.1mM and 10µM  $\text{Cu}^{2+}$  respectively; lane 6 was MES buffered with 1mM  $\text{Cu}^{2+}$ ; lane 7 was Tris buffered with 10mM EDTA; lane 8 was Tris buffered with 2.5mM  $\text{Cu}^{2+}$  and Complete™ protease inhibitor. Lanes 9 to 11 show immunodetection of long equivalent protein loadings with an anti-FLAG monoclonal antibody. They are all Tris buffered and contain 2.5mM  $\text{Cu}^{2+}$  1mM  $\text{Cu}^{2+}$ , and 10mM EDTA respectively. Lane 12 contains a 1µg protein loading silver stained gel of a Tris buffered cleavage reaction containing 1mM  $\text{Cu}^{2+}$ .

25

30

**Figure 4** Effect on extent of cleavage of Cu(II) concentration.

35

**Figure 5** Effect of metal ion on cleavage efficiency.

Coomassie stained non-reducing SDS-PAGE using 4-20% Tris-glycine gels. The size and migration of molecular weight markers are shown on the left in kDa. All lanes contain 1µg protein equivalents from cleavage reactions using 50mM of Tris buffer at pH9.0 for 24h at 62°C, and contain 1mM Cu<sup>2+</sup>, Fe<sup>3+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> and 2.5mM Cu<sup>2+</sup> in lanes 1 to 7 respectively.

**Figure 6** Effect of duration of incubation on cleavage efficiency.

**Figure 7** Effect of temperature of incubation on cleavage efficiency.

**Figure 8** HPLC mobility of cleaved and uncleaved CUT1 peptide.

**Figure 9** HPLC mobility of peptides collected and sequenced after cleavage of Null 2 FLAG F(ab')<sub>2</sub>

**Figure 10** Plasmid map for pDPH76.

## **EXAMPLES**

The following sections describe aspects of the invention in more detail. The following abbreviations are used: Fab' - antigen binding antibody fragment; F(ab')<sub>2</sub> - dimeric Fab'; LC - light chain; HC - heavy chain.

In the following, the use of Cu<sup>2+</sup> as an alternative to enzymatic cleavage of fusion proteins is demonstrated using a humanised γ1 Fab' with a C-terminal FLAG peptide as a model protein. A <sup>N</sup>DKTHC site was introduced between the hinge and the FLAG peptide after mutagenesis of the native <sup>N</sup>DKTHC sequence in the upper hinge to create a "null" site no longer cleaved by Cu<sup>2+</sup>.

### **Experimental protocol**

#### **Construction of 'Null 2 FLAG' Expression Plasmid**

Plasmid pDPH76 encoding 'Fab 40.4 hinge 1 Δ inter Null 2 FLAG' was derived from plasmid pDPH40 which encodes 'Fab 40.4 hinge 1/2 Δ inter'. pDPH40 has a *Spe* 1 restriction site introduced at the 3' end of the HC cistron - where the interchain disulphide Cys had been mutated to a Ser,

along with an adjacent Ser to Thr change (Humphreys DP *et al*, 1997). This enabled cloning of the region encoding the hinge, Cu<sup>2+</sup> site, spacer and FLAG as an *Spe* I-*Eco* RI oligonucleotide cassette after annealing the oligonucleotides:

- 5 5' TAGTCTGCAGGTGCTGACCTGCCCGCCGTGTCCGGATAAAACCCA  
TACCATCGAAGGCAGTACTAGCGATTATAAAGATGATGATGATAAATG  
ATGAGGATCCAAGCTTGCGGCCGCG 3', and 5' AATTCGCGGCCGCA  
AGCTTGGATCCTCATCATTTATCATCATCTTTATAATCGCTAGTAC  
10 TGCCTTCGATGGTATGGGTTTTATCCGGACACGGCGGGCAGGTCAG  
CACCTGCAGA 3' resulting in plasmid pDPH76, (Figure 10). The newly  
constructed coding region also contained unique *Bsp*E I and *Sca* I  
restriction sites. Codons preferred by *E. coli* were chosen [Wada KN *et al*,  
(1991)]. The sequence of the oligonucleotide encoded region was  
15 confirmed by sequencing of both strands using PRISM cycle sequencing  
kit and an ABI 373A sequencing machine.

#### **Production and Purification of 'Null 2 FLAG' F(ab')<sub>2</sub>**

- High density bacterial fermentations of strain W31 10 bearing pDPH76 were performed as described previously [Humphreys DP *et al* (1997)].
- 20 Extraction of periplasmic material, Protein G purification of Fab', F(ab')<sub>2</sub> production and Phenyl Sepharose purification were performed as described previously [Humphreys *et al*, (1998)]. After purification of F(ab')<sub>2</sub>, the protein was concentrated and buffer exchanged with several volumes of PBS using an Amicon pressurised stirred cell with a 10kDa
- 25 cut-off membrane to concentrations between 0.5 and 1.6mgml<sup>-1</sup>, and sterilised with a 0.22µm filter for storage at 4°C.

#### **Cu<sup>2+</sup> Cleavage Reactions**

- F(ab')<sub>2</sub> was included in all reactions at 0.33mgml<sup>-1</sup>, Tris and other buffers were used at a final concentration of 50mM (from a 500mM stock solution).
- 30 Buffers were made to pH with HCl, and Tris was used at pH 9.0 unless otherwise stated. All metal ions tested were chloride salts dissolved in dH<sub>2</sub>O and were used from 10x stocks for each concentration tested. Reactions were made up to volume with dH<sub>2</sub>O in 0.5ml eppendorf tubes,
- 35 vortex mixed and centrifuged briefly. Reaction volumes were most commonly 15µl, but were also scaled up to 150µl. In order to eliminate

evaporation during lengthy incubation at temperatures ranging from 59°C to 64°C reactions were covered in a layer of paraffin oil. Incubations at 62°C were in an air incubator, whilst other temperatures were in a Biometra TRIOThermoblock with heated lids. For SDS-PAGE 1µg samples were withdrawn through the paraffin oil for immediate electrophoresis or into 10mM EDTA and stored at 20°C prior to electrophoresis during time course experiments. Samples destined for HPLC analysis had paraffin oil removed by pipetting and ether extraction. EDTA free Complete™ protease inhibitor (Boehringer Mannheim, U.K.) was used as per manufacturers instructions.

#### **SDS-PAGE Analysis of 'Null 2 FLAG' Cleavage by Cu<sup>2+</sup>**

1µg per lane of Cu<sup>2+</sup> treated samples were electrophoresed on 4-20% Tris-glycine gels (Novex, U.K.) after boiling in non-reducing SDS-PAGE loading buffer for 3min. Gels were stained for 10min with Coomassie brilliant blue, and then destained before analysis by laser scanning densitometry on a Molecular Dynamics model 300A machine using ImageQuANT software version 4.2 or drying between cellulose membranes (Novex, U.K.). 'Null 2 FLAG' F(ab')<sub>2</sub> lacks the interchain disulphide bond and so separates into di-HC and free LC species during non-reducing SDS-PAGE. Since each F(ab')<sub>2</sub> molecule contains two Cu<sup>2+</sup> cleavage sites, three species are possible after incubation with Cu<sup>2+</sup>: Uncut (U), singly cleaved (S), and doubly cleaved (D) (Figure 3). Species with FLAG tails removed migrate more rapidly than uncut species during SDS-PAGE making them easy to identify (Figure 3). The lower band representing doubly cleaved species (D) is not immunoreactive to a anti-FLAG antibody, demonstrating that both FLAG tails have been removed from this species (lanes 9 and 10, Figure 3). F(ab')<sub>2</sub> treated with 10mM EDTA shows only singly cleaved species by immuno-detection even after a 24h incubation at 62°C (lane 11, Figure 3). Cleaved FLAG tail could be visualised after silver staining of SDS-PAGE (band F lane 12, Figure 3).

Percent cumulative cleavage was calculated after measurement of the area of absorbance for each of the peaks for the three species thus: cumulative cleavage

$$\frac{(\text{absorbance (S)} + \text{absorbance (D)})}{(\text{absorbance (U)} + \text{absorbance (S)} + \text{absorbance (D)})} \times 100$$

Gels were silver stained with 'Silver Stain II' from Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan. Western blotting was performed as described previously [Humphreys DP *et al*, (1997)], except that the primary antibody used was anti-FLAG monoclonal M2 at 1/500 dilution (Kodak, U.K.), which was revealed with ExtrAvidin-HRP (Sigma) at 1/500. 'Rainbow' markers were used as molecular weight standards throughout (Novex, U.K.).

### **Surface Plasmon Resonance**

Kinetic analysis to determine the on and off rates for antigen binding to 'Null 2 FLAG' F(ab')<sub>2</sub> molecules was performed using a BIACORE 2000 (Biacore AB). 'Null 2 FLAG' F(ab')<sub>2</sub> molecules were captured by an anti-human IgG, which is immobilised on the sensor chip surface, followed by an injection of soluble antigen. Affinipure goat anti-human Ig, F(ab')<sub>2</sub> fragment specific (Jackson ImmunoResearch) was immobilised on a Sensor Chip CM5 via amine coupling chemistry to a level of 15500RU. HBS buffer (10mM HEPES pH7.4, 0.15M NaCl, 3mM EDTA, 0.005% Surfactant P20, Biacore AB) was used as the running buffer with a flow rate of 10µl/min. An injection of 'Null 2 FLAG' F(ab')<sub>2</sub> was captured by the immobilised anti-human IgG to a level between 340-390RU. Soluble antigen was injected over the captured 'Null 2 FLAG' F(ab')<sub>2</sub> surface at 2 and 0µg/ml. The surface was regenerated by injecting 2 x 5µl of 30mM HCl. The sensorgram for soluble antigen binding was corrected with the control buffer sensorgram. Kinetic parameters were calculated using BIA evaluation 2.1 software.

### **Mass Spectrometry**

Molecular mass for Fab' and F(ab')<sub>2</sub> was determined using Fisons VG Quattro 1 triple quadrupole equipment in electrospray ionisation mode. F(ab')<sub>2</sub> samples were desalted to remove Tris by multiple volume exchanges with 10mM ammonium acetate using Microcon concentrators with a 10kDa membrane cutoff size (Amicon, U.K.).

### **Amino Acid Composition Analysis**

This was carried out at the Dept. of Mol. and Cell Biol., University of Aberdeen, U.K. on a ABI 420A machine after hydrolysis with 6M HCL for 40min at 160°C under Argon.

5

### **HPLC purification and N-terminal sequencing of cleaved FLAG tail from F(ab')<sub>2</sub>.**

HPLC purification - reaction mixtures containing cleaved/uncleaved F(ab')<sub>2</sub> were resolved by high performance liquid chromatography, using an Aquapore RP300, 7 micron, 2.1 x 100mm column on a Hewlet Packard HP1090 with on-line diode array for spectral analysis. Components were resolved with a linear gradient of 2 to 95% acetonitrile in water/trifluoroacetic acid (0.1% v/v), at a flow rate of 0.5 ml/min, at 21°C. Peaks absorbing at 214nm were collected and concentrated under vacuum to remove acetonitrile for peptide sequencing.

15

Peptide sequencing - peptides in fractions were bound to polyvinylidene difluoride membrane (Prosorb, Applied Biosystems), and 100mg polybrene (Biobrene, Applied Biosystems) applied to, and air-dried on, the membrane. Each peptide on Prosorb membrane was then analysed by sequencing on an Applied Biosystems 470A with on-line 120A analyser.

20

### **HPLC analysis of peptide cleavage by Cu<sup>2+</sup>**

Peptides were dissolved in dH<sub>2</sub>O and used at 0.5mM final concentrations in incubations with Tris pH9.0 at 50mM, and CuCl<sub>2</sub> from 2.5mM to 100µM made up to final reaction volume of 20µl with dH<sub>2</sub>O in 0.5ml eppendorf tubes, vortex mixed and centrifuged briefly before overlaying with paraffin oil and incubating at 62°C for 16-24h. Samples had paraffin oil removed by pipetting and ether extraction. Peptides were analysed by HPLC, using a Symmetry C18 column (3.5 micron, 4.6 x 150mm) on a Hewlet Packard HP1090 with on-line diode array for spectral analysis. The components of the sample were resolved on a linear gradient, from 5 to 95% acetonitrile in trifluoroacetic acid (0.1% v/v), at a flow rate of 0.5ml/min, at 21°C. Elution was monitored at 214nm.

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## RESULTS

### Engineering of Fab' with C-terminal Cu<sup>2+</sup> cleavage site.

The Fab' was derived from a previously described humanised  $\gamma$ 1 Fab' ('Fab 40.4 hinge 1  $\Delta$  inter') [Humphreys DP *et al*, (1997)] This binds a human cytokine with high affinity and is purified in high yield from the periplasm of *E.coli* after high cell density fermentation of strains bearing Fab' expression plasmid. The 'Fab 40.4 hinge 1  $\Delta$  inter' has two cysteines in an intact  $\gamma$ 1 hinge and lacks the inter light chain-Fd disulphide bond (hereafter, interchain), due to mutagenesis of the interchain cysteines to serines. In order to demonstrate that the 'null' Cu<sup>2+</sup> site was not cleaved by Cu<sup>2+</sup>, the Fab' was dimerised to F(ab')<sub>2</sub>. Non-reducing SDS-PAGE of F(ab')<sub>2</sub> lacking the interchain bond produces a readily identifiable di-heavy chain species with a mass of ~50kDa which can remain dimeric only if the 'null' site is not cleaved by Cu<sup>2+</sup>. The smaller size of the di-HC species compared to the F(ab')<sub>2</sub> species also facilitated accurate analysis of the extent of protein cleavage by non-reducing SDS-PAGE.

All four residues in the native NDKTH<sup>C</sup> sequence of the upper hinge of 'Fab 40.4 hinge 1  $\Delta$  inter' were mutated in order to destroy the Cu<sup>2+</sup> cleavage site, resulting in the protein termed 'Null 2 FLAG'. Following the middle hinge there is a short hydrophilic/flexible spacer region, followed by a Cu<sup>2+</sup> cleavage site NDKTH<sup>C</sup>, and finally a FLAG peptide [Brizzard BL *et al*, (1994)]. The C-termini sequences of the two heavy chains are shown below for comparison.

25

'Fab 40.4 hinge 1  $\Delta$  inter' heavy chain

N . .EPKTSDKTHTCPPCPA<sup>C</sup>

Cu<sup>2+</sup>

30

'Fab 40.4 hinge 1  $\Delta$  inter Null 2 FLAG' heavy chain

N . .EPKTSLQVLTCPPCDKTHIEGSTSDYKDDDDK<sup>C</sup>

Null2

Cu<sup>2+</sup>

FLAG

The 'Null 2 FLAG' Fab' was found to be intact after purification from *E.coli* cell fermentation cell pastes by Protein G chromatography by mass spectrometry. The observed masses for light and heavy chain were



23,382.66  $\pm$  1.18 and 26,739.62  $\pm$  1.87 respectively, compared to the predicted masses of 23,384.25 and 26,742.04 respectively. Hence, the introduced Cu<sup>2+</sup> site, flexible spacer, and FLAG peptide were not cleaved by *E.coli* proteases or media components.

5

#### **Effect of buffer composition and pH.**

It was known that the cleavage reaction of the native upper hinge site was favoured by alkaline pHs [Smith MA *et al*, (1996)]. A strong buffer was needed to enable investigation of the optimal pH since concentrated CuCl<sub>2</sub> is acidic. Buffers with primary amines are known to have a binding capacity for Cu<sup>2+</sup> ions [Dawson RMC *et al*, (1989)]. We tested the efficiency of cleavage reactions using 50mM Tris, Bicine, Tricine, CHES, and HEPES as buffers at pH 8.5 using Cu<sup>2+</sup> concentrations of 1mM and 100 $\mu$ M. Surprisingly, Tris resulted in the most efficient cleavage, Bicine and Tricine minimal cleavage, whilst CHES and HEPES resulted in loss of F(ab')<sub>2</sub> material (Figure 1). The Cu<sup>2+</sup> binding capacity of the Tris buffer (which contains 1<sup>o</sup> amines) may actually be beneficial, acting as a Cu<sup>2+</sup> chelation/buffer system and by presentation of Cu<sup>2+</sup> to the Fab' Cu<sup>2+</sup> binding site.

20

Tris was then tested at pHs 8.0, 8.5, 9.0, 9.25, and 9.5 to define the optimal pH for cleavage reactions. The results in Figure 2 show clearly an increase in protein cleavage with increasing pH. pH 9.0 was chosen for all subsequent cleavage reactions as a compromise between efficient cleavage, effective buffering, and the desire to use mild incubation conditions. Incubation with 0.1mM, 1mM, and 2.5mM Cu<sup>2+</sup> at pH 5.5 in MES buffer did not result in cleavage of the FLAG tail (lanes 11 and 12 Figure 1, and lane 6 Figure 3)

#### **Effect of Cu<sup>2+</sup> Concentration.**

The [Cu<sup>2+</sup>] giving optimal protein cleavage was determined empirically. Maximum protein and peptide cleavage was found previously to be at a molar ratio of between 0.5 and 1 Cu<sup>2+</sup>:protein/peptide in a non-Tris buffer [Smith MA *et al*, (1996) and Allen G & Campbell RO(1996)]. Since Tris buffer has a significant Cu<sup>2+</sup> binding capacity, prediction of the effective maximum [Cu<sup>2+</sup>] was difficult. A range of Cu<sup>2+</sup> concentrations from 10mM

35

to 10 $\mu$ M were tested. 10mM and 7.5mM Cu<sup>2+</sup> were found to cause immediate and overnight precipitation respectively, but the SDS-PAGE analysis for the remainder of the titration are shown in Figure 3. Quantification of the effect of [Cu<sup>2+</sup>] is shown in Figure 4. This shows that there is a clear dependence of the extent of cleavage upon [Cu<sup>2+</sup>]. Such cleavage was completely inhibited by inclusion of EDTA at a final concentration of 10mM (lane 7, Figure 3). The extent of cleavage with 2.5mM Cu<sup>2+</sup> was unaffected by inclusion of a broad range protease inhibitor (lane 8 Figure 3), suggesting that this highly purified F(ab')<sub>2</sub> sample was not being subjected to the effects of a contaminating protease. No cleavage of 'Null 2 FLAG' is seen after 24hr incubation with Ca<sup>2+</sup>, Fe<sup>3+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, or ions Zn<sup>2+</sup> at 1mM (Figure 5), demonstrating that the cleavage event was due specifically to the presence of Cu<sup>2+</sup> ions.

#### 15 **Effect of Duration of Incubation.**

The dynamics of cleavage at 62°C with 2.5mM Cu<sup>2+</sup> are non-linear (Figure 6). There was a short lag phase of approximately 2h before significant protein cleavage was observed. Cleavage proceeded fairly linearly until approximately 12-14 hours (~50% cleavage), followed thereafter by a slowing in the accumulation of cleaved species. After an overnight incubation with 2.5mM Cu<sup>2+</sup> approximately 53% of Cu<sup>2+</sup> sites had been cleaved, 77% after a 24h incubation, and reaching 86% after 28.5h. The lag phase may be an indicator of a Cu<sup>2+</sup> availability effect - equilibration of Cu<sup>2+</sup> between Tris and F(ab')<sub>2</sub> binding sites, or generation of reactive peptide cleaving species [Allen G and Campbell RO (1996); Wolff SP *et al*, (1986); and Garrison WM (1968)]. The decrease in cleavage rate after 14h probably simply reflects the decreasing availability of uncleaved substrate.

#### 30 **Effect of Temperature**

The effect of temperature of incubation on the efficiency of cleavage by Cu<sup>2+</sup> was investigated by following a time course of cleavage at 59°C, 61°C, and 64°C, allowing comparison with that observed at 62°C. The results (Figure 7) show that increasing the temperature increases both the initial rate of accumulation of cleaved species and the final total percent cleavage after a 28.5h incubation. However, at 64°C protein loss was

observed during the incubation, resulting in a sharp decrease in cleaved species and a concomitant increase in measurement errors and loss of LC.

- 5 The particular maximum tolerated temperature in a  $\text{Cu}^{2+}$  cleavage reaction will depend upon substrate protein type and concentration and  $[\text{Cu}^{2+}]$  buffer type and concentration. We observe a small but significant amount ( $\approx 25\%$ ) of 'Null 2 FLAG' loss at  $62^\circ\text{C}$  toward the end of the incubation, but non after incubation at  $59^\circ\text{C}$  for 28.5h.

10

Lack of the interchain disulphide bond in 'Null 2 FLAG' may play an important role in the temperature sensitivity of this protein during prolonged incubation at elevated temperatures. The LCs and HCs of this  $\text{F(ab')}_2$  can presumably part transiently in solution, and the rate of this

15 parting will increase with temperature. Exposure of normally buried surfaces, particularly on the HC may lead to protein:protein and protein:tube aggregation events.

#### **Specificity of $\text{Cu}^{2+}$ Catalysed Protein Cleavage**

- 20 In order to discover whether 'Null 2 FLAG' was being cleaved as expected between the Lys and Thr of the introduced  $\text{NDKTH}^{\text{C}}$  sequence, a sample of 'Null 2 FLAG' cleaved with 2.5mM  $\text{Cu}^{2+}$  for 18 hours was analysed by electrospray mass spectrometry. Depending upon the extent of cleavage each di-HC species can have one or both FLAG tails removed, resulting in
- 25 species S and D as seen in Figure 3 (singly and doubly cleaved respectively). Cleavage between Lys and Thr would result in species with predicted masses of 51573.35 and 49664.62 for the singly and doubly cleaved species respectively. We find that the two strongest peaks relating to di-HC had observed masses of  $51565.68 \pm 10.47$  and  $49662.08$
- 30  $\pm 11.16$ , which are in agreement with the predicted masses for di-HC with one and two FLAG tails removed. In addition, we find no evidence of the species that would be generated if the di-HC were cleaved at the 'Null 2' site, nor for non-specific  $\text{Cu}^{2+}$  cleavage fragments.
- 35 To confirm that the 'Null 2' sequence was not cleavable we tested the effect of incubation of the peptides 'NULL 2' ( $\text{N Ac-VEPKTSLQVLT-NH}_2$ )

and 'CUT 1' ( $N$  Ac-VEPKTSDKTHT-NH<sub>2</sub>) with Cu<sup>2+</sup> in 50mM Tris pH 9.0 at 62°C. The peptide 'NULL 2' was not cleaved by Cu<sup>2+</sup> from 0.1mM to 2.5mM (data not shown), whereas 'CUT 1' was seen to be cleaved by the higher concentration of Cu<sup>2+</sup> (Figure 8). There is a clear change in the mobility and distribution of the two peaks that represent CUT1 peptide after treatment with 2.5mM Cu<sup>2+</sup>, however we cannot explain the unusual doublet migration of this peptide during HPLC. Pure CUT1 was found by mass spectrometry to be  $\geq 95\%$  full length material (mass 1282.5). After incubation of CUT1 with 2.5mM Cu<sup>2+</sup>, collection of peaks from HPLC purification, and mass spectrometry show the presence of a major and a trace species with molecular masses of 944.5 and 1281.8 respectively. CUT1 peptide cleaved between the Lys and Thr of the cleavage site (Ac-VEPKTSDK-OH) has a predicted mass of 944.48, whilst the full length and unmodified CUT1 peptide has a predicted mass of 1282.5 demonstrating that the change in peaks shown for the Cu<sup>2+</sup> treated material in Figure 8 represent correct cleavage of CUT1 peptide.

N-terminal amino acid sequencing of HPLC purified Cu<sup>2+</sup> cleaved FLAG tail was performed to ascertain the integrity of the cleavage site amino acids. Fractions relating to four peptide peaks with different migration properties during HPLC purification were collected and sequenced (Figure 9). All were found to have the correct N-terminal NTHTIEG<sup>C</sup> sequence confirming accurate cleavage at the predicted site, and that the Thr and His residues in the Cu<sup>2+</sup> site had not been destroyed or modified during the reaction. The two most abundant fractions were collected at the start of the elution gradient and gave complete sequence of NTHTIEGSTSDYKDDDDK<sup>C</sup>. The reason for the heterogeneity of FLAG tail peptide migration during HPLC is not known.

### 30 **Effect of Cleavage Conditions on Protein Function**

SDS-PAGE and mass spectrometry analysis showed that the cleavage conditions had not grossly affected the integrity of the 'Null 2 FLAG' protein, as witnessed by the absence of new protein fragments. We took advantage of the antigen binding ability of the Fab 40.4 derived 'Null 2 FLAG' to investigate whether more subtle changes in the protein occur after cleavage reaction incubation. Modification of residues in the CDRs

might well result in a loss of antigen binding. Gly, Pro, Lys, and His in particular are known to be sites for protein damage by divalent metal ions [Dean RT *et al*, (1989); Easton CJ *et al*, (1997); Hawkins CL and Davies MJ (1997)]. The CDRs of 'Null 2 FLAG' contain a total of 5 Gly, 2 Lys, and 1 Pro residues, so we might expect that any non-specific damage to the F(ab')<sub>2</sub> might be reflected more measurably in loss of affinity due to damage specifically to the CDRs. Surface plasmon resonance affinity measurements (Table I) show that overnight incubation at pH 9.0 with or without 2.5mM Cu<sup>2+</sup> do not significantly affect the affinity of 'Null 2 FLAG' F(ab')<sub>2</sub>. The very minor apparent loss of affinity is actually caused by the incubation procedure, and not due to the Cu<sup>2+</sup> itself. This may be a reflection of the ability of the LC and HC of the F(ab')<sub>2</sub> to part, due to the absence of the interchain disulphide bond.

**Table 1**

Treatment	k <sub>ass</sub> (M)	k <sub>ass</sub> (M)	kD (M)
50mM Tris pH 9.0, 62°C, 16h, 2.5mM Cu <sup>2+</sup>	4.22 x 10 <sup>-5</sup>	2.08 x 10 <sup>-4</sup>	4.93 x 10 <sup>-10</sup>
50mM Tris pH 9.0, 62°C, 16h, 10mM EDTA	4.27 x 10 <sup>-5</sup>	1.85 x 10 <sup>-4</sup>	4.33 x 10 <sup>-10</sup>
Untreated	3.93 x 10 <sup>-5</sup>	1.28 x 10 <sup>-4</sup>	3.26 x 10 <sup>-10</sup>

In addition, we performed a total amino acid composition analysis on 'Null 2 FLAG' F(ab')<sub>2</sub> that had been incubated for 16h at 62°C in 50mM Tris pH 9.0 with or without 2.5mM Cu<sup>2+</sup>. There were no new peaks in the Cu<sup>2+</sup> treated sample that might be evidence of modified amino acids, and no significant difference in the amino acid composition between the two samples as evidence of degradation of particular amino acids.

## Discussion

The Results show evidence for use of the tetra-peptide sequence <sup>N</sup>DKTHC as a site for specific cleavage of fusion proteins by cupric ions. In the model protein a C-terminal FLAG peptide is cleaved off exactly between

the Lys and Thr of the target sequence as shown by mass spectrometry of the remaining N-terminal F(ab')<sub>2</sub> Part, and N-terminal sequencing of liberated C-terminal FLAG peptide.

- 5 The cleavage is efficient, reaching levels of 71%, 81%, and 86% after 28.5h incubations at pH9.0, and 59°C, 61°C, 62°C respectively. The cleavage site does not need to be dimeric in order to be cleaved, since we see significant accumulation of F(ab')<sub>2</sub> species with both one and two FLAG tails removed. We find no evidence for gross deleterious effects on
- 10 the structure or activity of the F(ab')<sub>2</sub> used in this system, even after incubation with 2.5mM Cu<sup>2+</sup> for 16h at 62°C, pH 9.0. It is clear that the high incubation temperature is the most critical element in determining the quality of protein after the cleavage treatment. With this particular protein the 5°C difference between 59°C and 64°C changes the incubation from
- 15 one that gives a yield of 71% cleavage and unmeasurable protein loss to one with ≥70 % protein loss after a 28.5h incubation. However, the maximum tolerated temperature for other proteins needs to be determined empirically.
- 20 The efficiency of cleavage reactions is high even without final optimisation of the cleavage process for 'Null 2 FLAG' F(ab')<sub>2</sub>. Simply by increasing the [Cu<sup>2+</sup>] from 2.5mM to ≤5mM, and the pH to 9.5 we could increase the extent of cleavage at any given time.
- 25 The 'Null' site of 'Null 2 FLAG' contains changes to all four residues (NDKTHC → NLQVLC). To minimise the risk of immunogenicity against Fab' material cleaved by this technique requires making a minimal number of changes in the native NDKTHC site whilst remaining uncleavable by Cu<sup>2+</sup>. The same four residues (D, K, T, and H) are involved in the metal
- 30 binding site of serum albumins, and so are clearly capable of chelating divalent cations [Sadler PJ *et al*, (1994)]. However, in the creation of a 'Null' site the relative effect of each residue upon the ability of the site to be cleaved, rather than simply chelate Cu<sup>2+</sup> is critical. The pH dependence of the cleavage reaction and peptide variants implicate the importance of the
- 35 His residue in the cleavage reaction [Allen Gand Campbell RO, (1996)]. It is possible that a single mutation of this residue, or combined with

mutation of the Thr residue to a small or hydrophobic residue might result in this remaining uncleavable by  $\text{Cu}^{2+}$ .

5 No evidence was found for cleavage of 'Null 2 FLAG' F(ab')<sub>2</sub> at sites other than that of the  $\text{NDKTH}^{\text{C}}$  sequence. This is consistent with the absence of this tetrapeptide from the 1<sup>o</sup> structure of 'Fab 40.4'. In fact we find that the  $\text{NDKTH}^{\text{C}}$  sequence is relatively rare. Only 167 matches are found against 256,226 entries searched in the NCBI protein database. To be cleaved by  $\text{Cu}^{2+}$  this sequence must be exposed, and probably also requires a  
10 flexible/disordered structure. This combination of rarity of both 1<sup>o</sup> and 3<sup>o</sup> structure of the  $\text{Cu}^{2+}$  cleavage site makes the target specific enough to be a general protein cleavage site. The protein retains only two amino acids of the site after cleavage, and so these are unlikely to affect its structure or function. If the cleavage site can be introduced where a Lys or His already  
15 exist, then the number of extraneous residues is reduced to one. This may be of more importance where potential immunogenicity is an issue.

The enormous benefit of using  $\text{NDKTH}^{\text{C}}/\text{Cu}^{2+}$  is in cost and convenience. Many proteases cannot be used for large or industrial scale processes due  
20 to the cost involved, and the problem of ensuring complete removal of the contaminating protease after cleavage. Assuming equal cleavage efficiencies, the cleavage of protein with  $\text{CuCl}_2$  is approximately  $1.2 \times 10^5$  fold cheaper than using Factor Xa protease. In addition, the  $\text{CuCl}_2$  is neither animal derived or produced using animal products removing the  
25 risk of viral/prion contamination and can be removed simply from the protein sample by addition of EDTA, followed by dialysis. Cleaved material should be in the flow through after re-loading onto an affinity column, giving a simple method of separating cleaved and uncleaved  
30 material when the fusion partner is an affinity tail.

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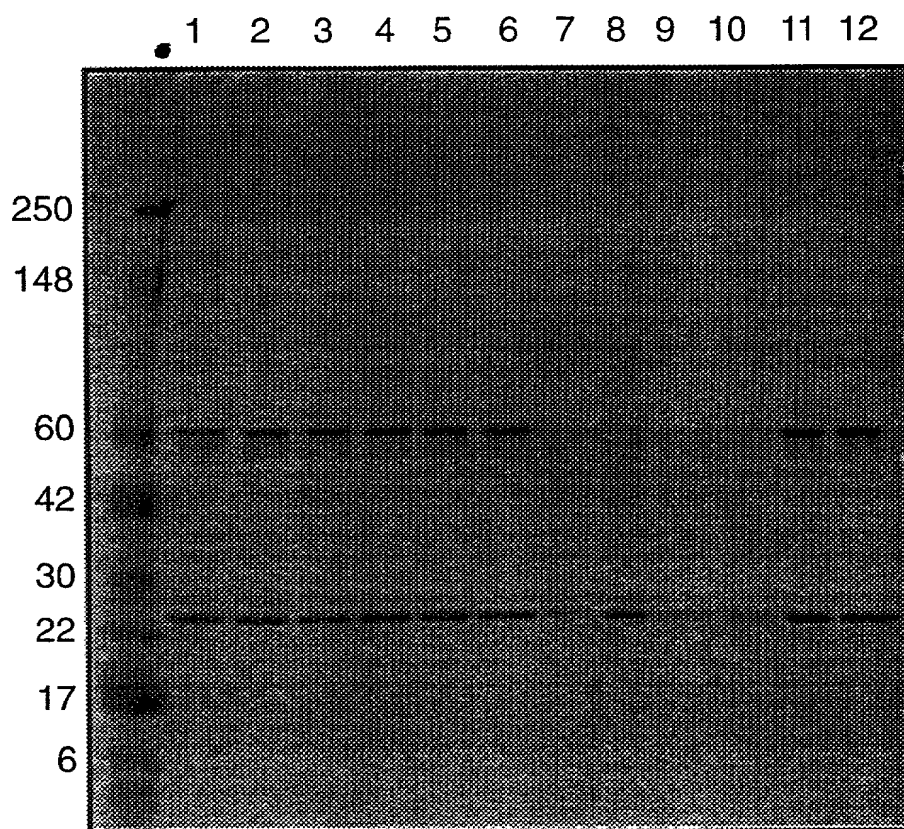
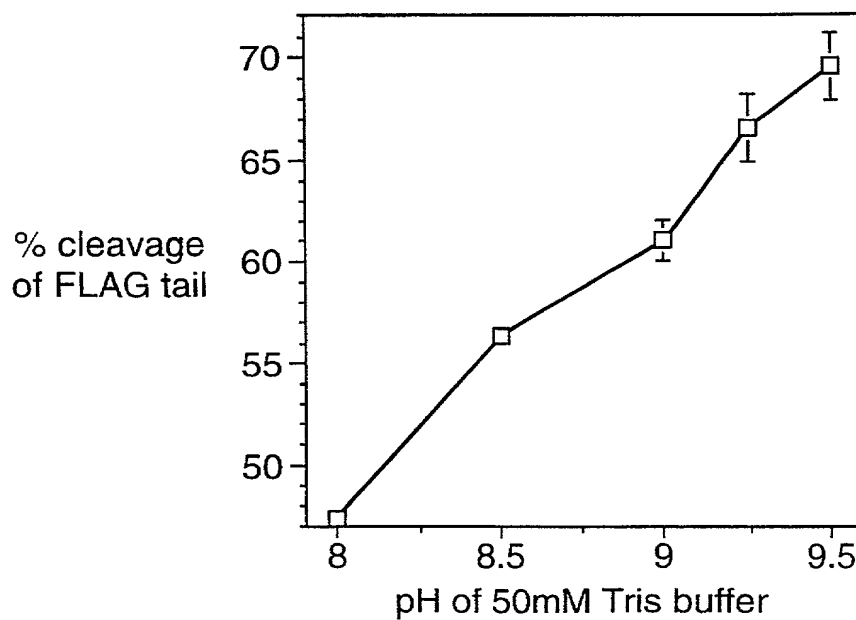


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**CLAIMS**

1. A DNA coding for a fusion protein comprising two polypeptides joined by a cleavage site which does not naturally join the polypeptides and which is specifically cleavable by copper(II) ions.  
5
2. DNA according to Claim 1 coding for a fusion protein in which the cleavage site is the tetrapeptide  $\text{NDKTH}^{\text{C}}$  or a functional equivalent thereof.  
10
3. DNA according to Claim 2 wherein the cleavage site is  $\text{NDKTH}^{\text{C}}$ ,  $\text{NDRSH}^{\text{C}}$ ,  $\text{NEKSH}^{\text{C}}$  or  $\text{NDKSH}^{\text{C}}$ .  
15
4. A vector comprising DNA according to Claim 1 to Claim 3.
5. A host cell transformed with a vector according to Claim 4.
6. A process for the productin of a desired protein in native form comprising 1) expressing the desired protein as a fusion protein in a host cell according to Claim 5 in which the desired protein is expressed as one of two polypeptides joined by a site specifically cleavable by copper(II) ions, 2) cleaving the fusion protein with copper(II) ions and 3) recovering the desired cleaved protein in native form.  
20
7. A process according to Claim 6 wherein the desired protein is an antibody or a fragment thereof.  
25

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**FIG. 1****FIG. 2** Effect of pH on efficiency of cleavage by Cu(II)

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FIG. 3

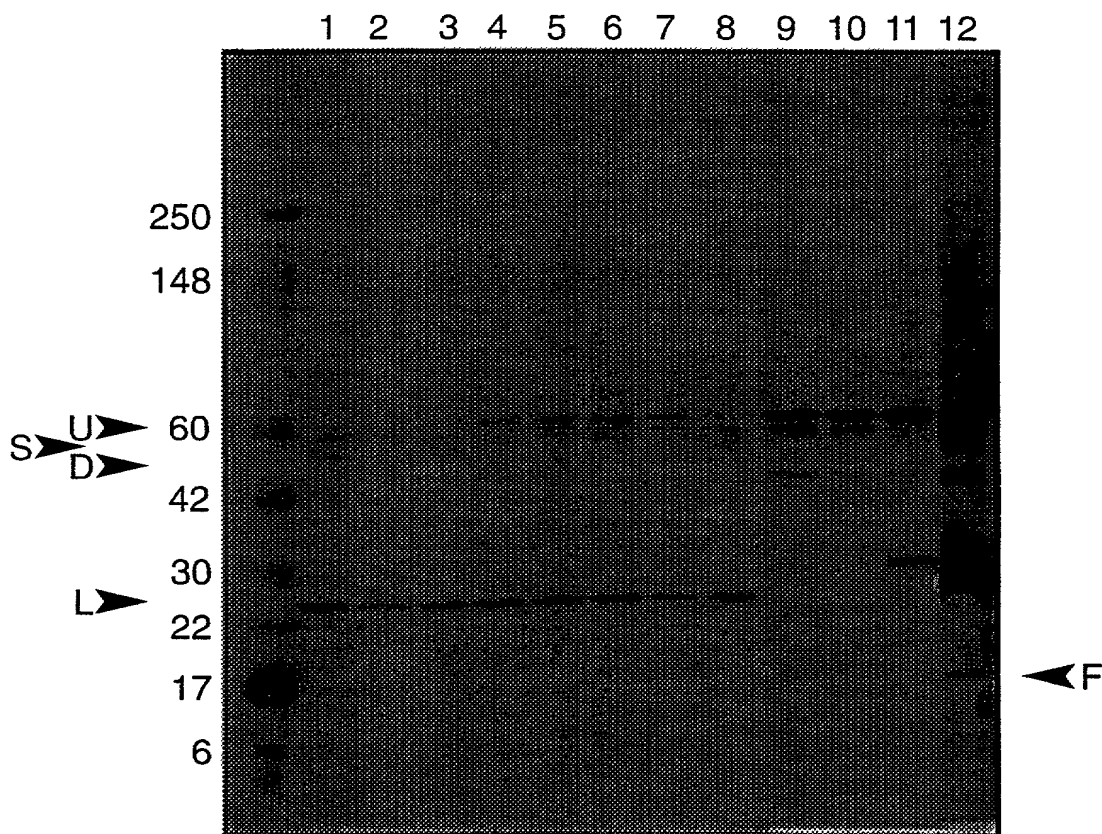
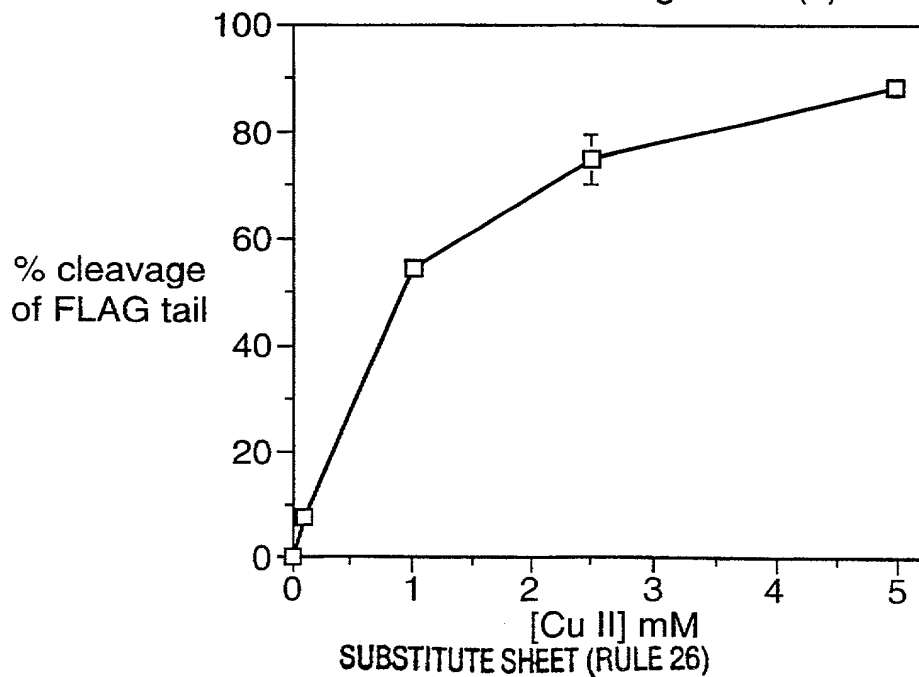


FIG. 4 Effect on extent of cleavage of Cu(II) concentration



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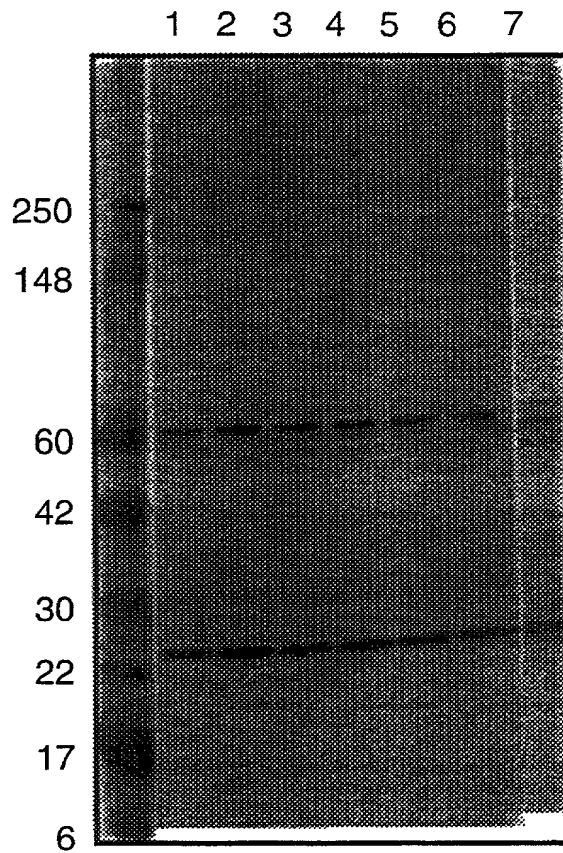
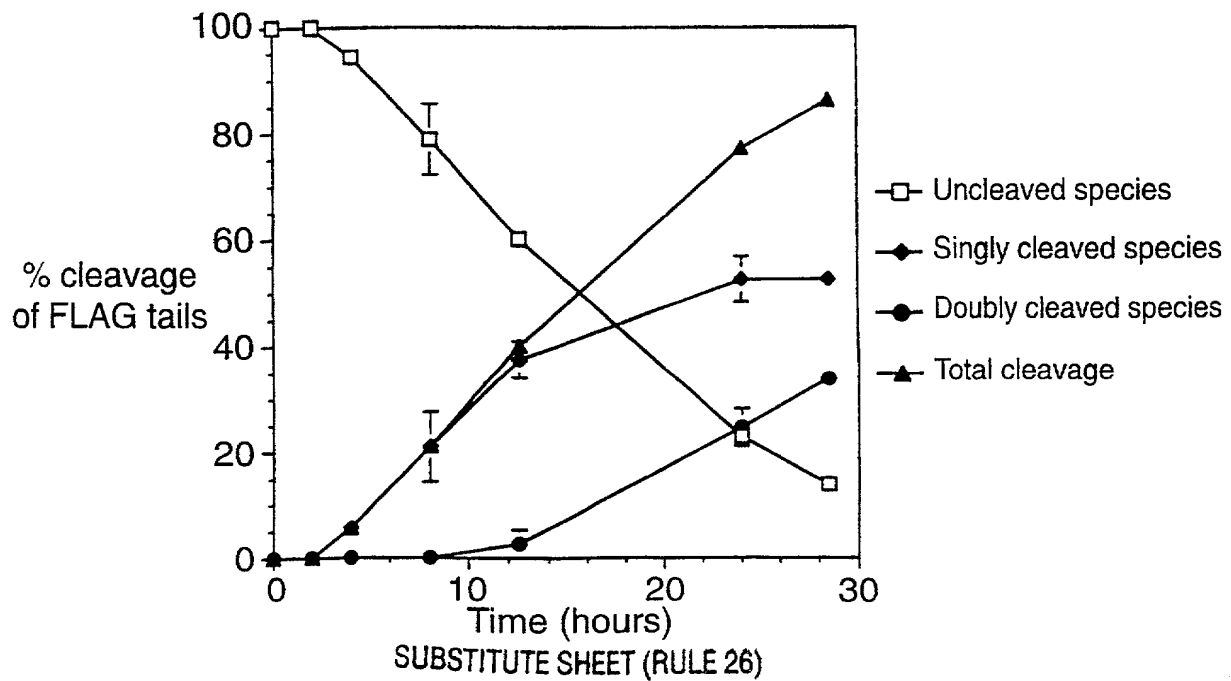
**FIG. 5****FIG. 6** Effect of duration of incubation on cleavage efficiency

FIG. 7

Effect of temperature of incubation on cleavage efficiency

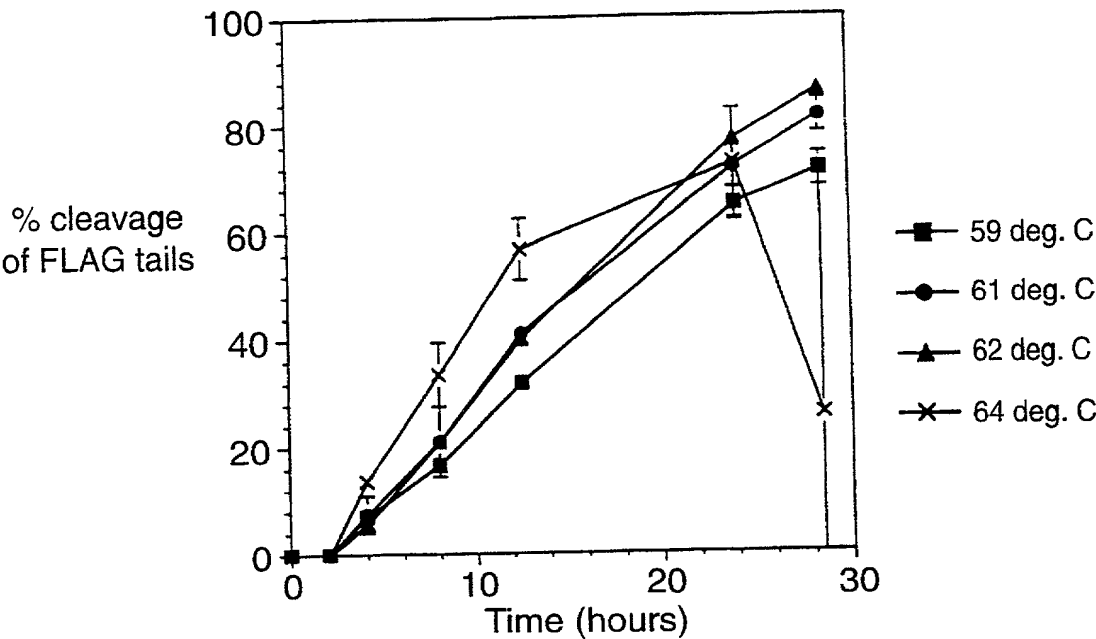
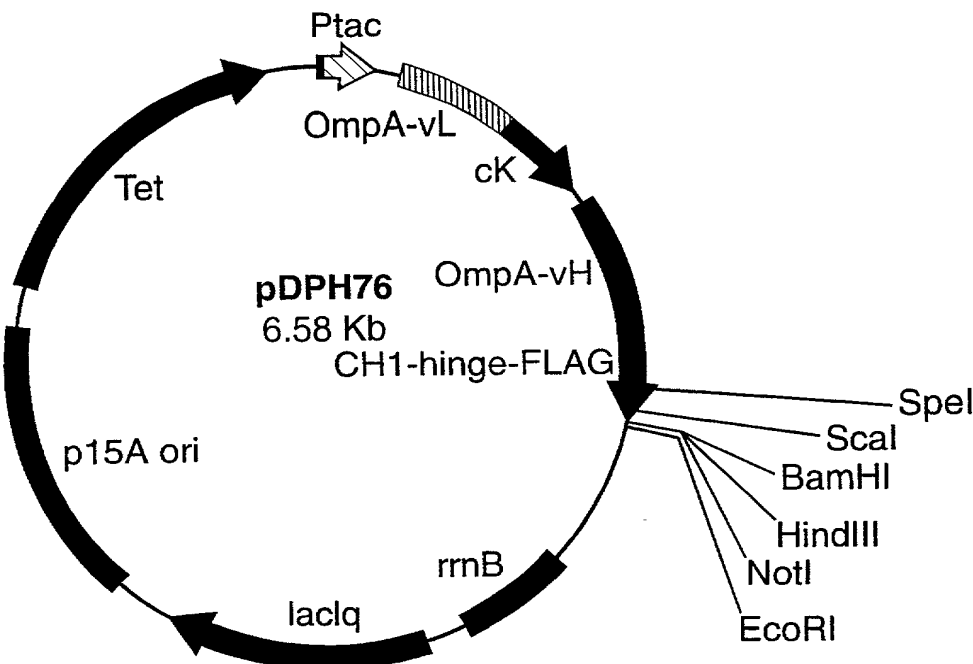
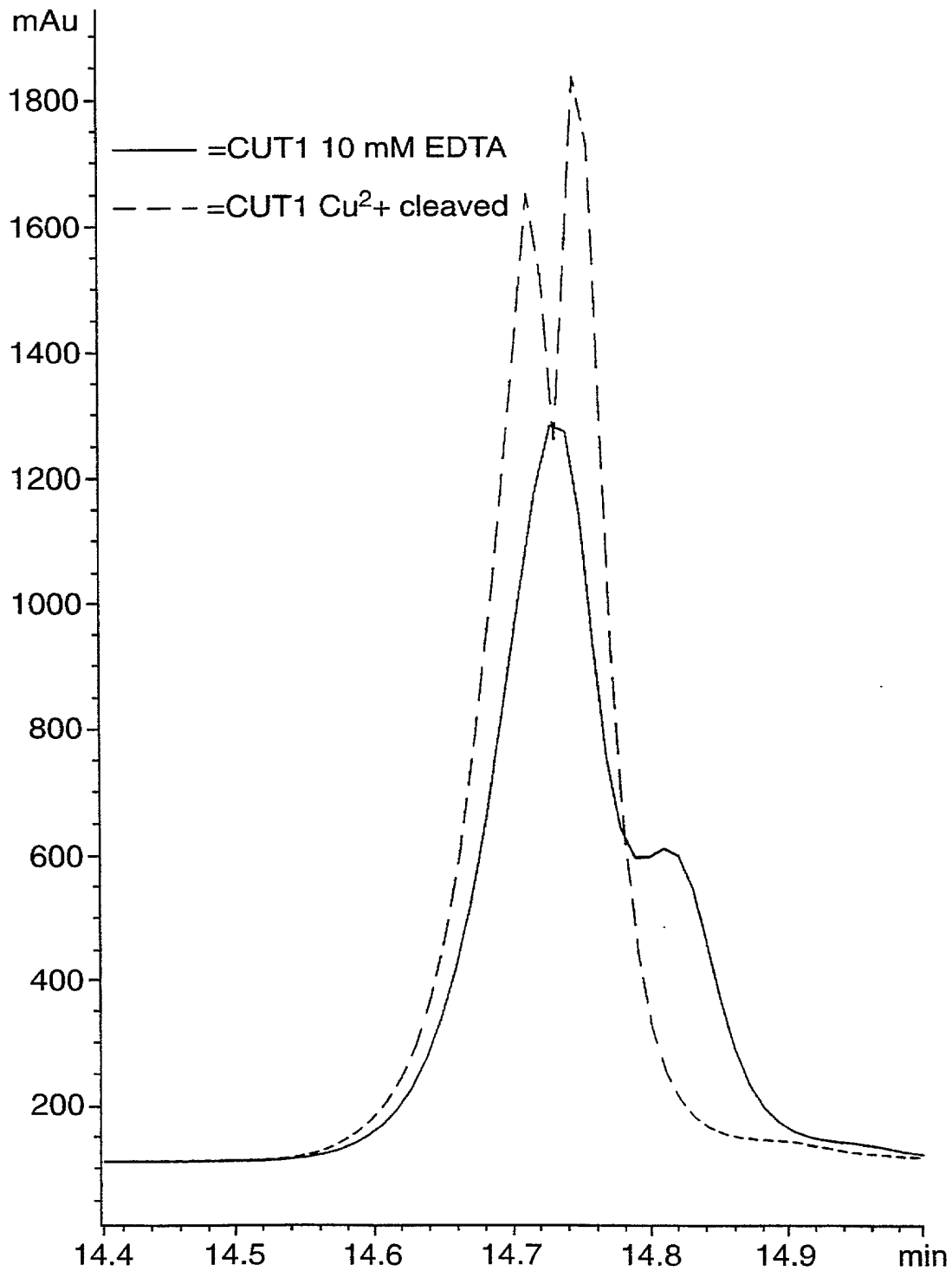


FIG. 10



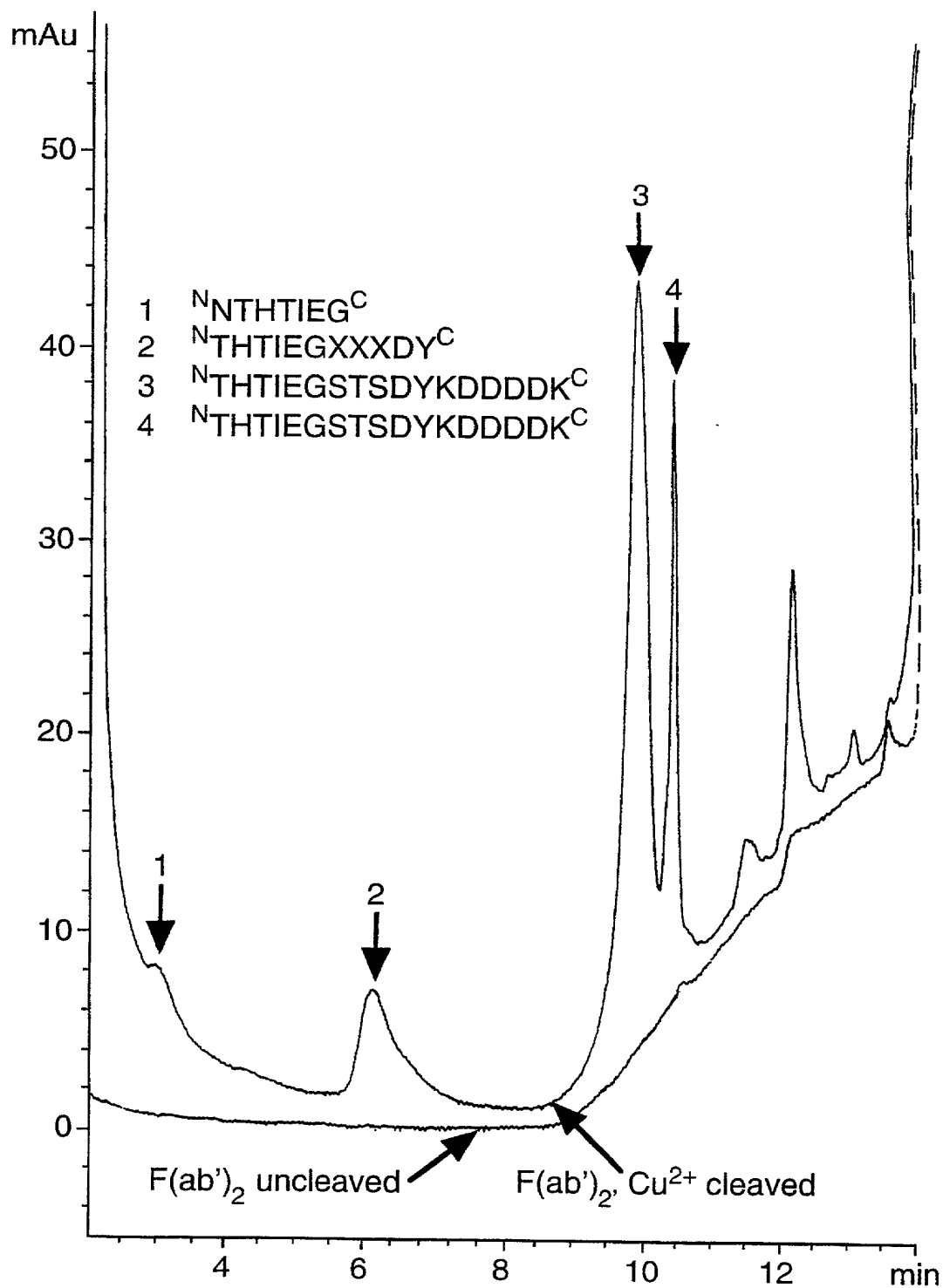
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FIG. 8



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FIG. 9



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**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below under my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**DNA ENCODING FUSION PROTEINS WHICH ARE SPECIFICALLY  
CLEAVABLE BY COPPER (II) IONS**

the Specification of which

☒ is attached hereto

☒ was filed on 29 November 1999

as International Application No. PCT/GB99/03979

I hereby state that I have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

<u>APPLICATION</u> <u>NUMBER</u>	<u>PRIOR FOREIGN FILED APPLICATION(S)</u> <u>COUNTRY</u> <u>(MONTH/DAY/YYYY)</u>	<u>PRIORITY</u> <u>CLAIMED</u>
9826112.6	Great Britain November 27, 1998	YES

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER(S)

FILING DATE (MM/DD/YYYY)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

<u>U.S. Parent Application No.</u>	<u>PCT Parent Number</u>	<u>Parent Filing (MM/DD/YYYY)</u>	<u>Parent Patent Number (if applicable)</u>
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The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from **Carpmaels & Ransford** as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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for the law firm of Klauber & Jackson, said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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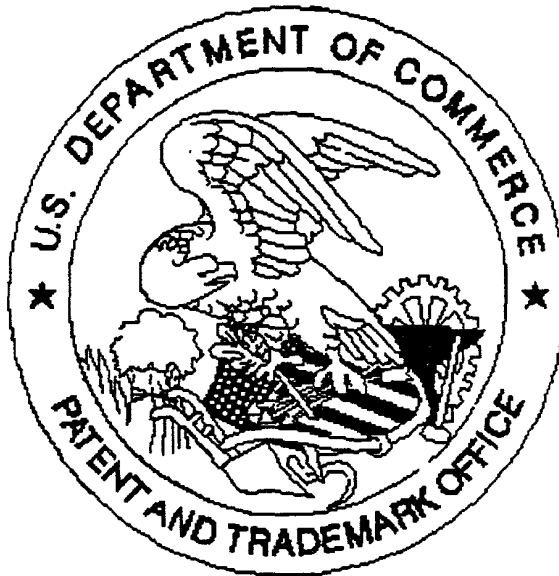
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SIGNATURE OF INVENTOR

*David Paul Humphreys*

DATE 7th June 2001

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